

Resolving the Function of Distinct Munc18-1/SNARE Protein Interaction Modes in a Reconstituted Membrane Fusion Assay^{*[5]}

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Sec1p/Munc18 proteins and SNAP receptors (SNAREs) are key components of the intracellular membrane fusion machinery. Compartment-specific v-SNAREs on a transport vesicle pair with their cognate t-SNAREs on the target membrane and drive lipid bilayer fusion. In a reconstituted assay that dissects the sequential assembly of t-SNARE (syntaxin 1-SNAP-25) and v-/t-SNARE (VAMP2-syntaxin 1-SNAP-25) complexes, and finally measures lipid bilayer merger, we resolved the inhibitory and stimulatory functions of the Sec1p/Munc18 protein Munc18-1 at the molecular level. Inhibition of membrane fusion by Munc18-1 requires a closed conformation of syntaxin 1. Remarkably, the concurrent preincubation of Munc18-1-inhibited syntaxin 1 liposomes with both VAMP2 liposomes and SNAP-25 at low temperature releases the inhibition and effectively stimulates membrane fusion. VAMP8 liposomes can neither release the inhibition nor exert the stimulatory effect, demonstrating the need for a specific Munc18-1/VAMP2 interaction. In addition, Munc18-1 binds to the N-terminal peptide of syntaxin 1, which is obligatory for a robust stimulation of membrane fusion. In contrast, this interaction is neither required for the inhibitory function of Munc18-1 nor for the release of this block. These results indicate that Munc18-1 and the neuronal SNAREs already have the inherent capability to function as a basic stage-specific off/on switch to control membrane fusion.

Membrane fusion in eukaryotic cells is mediated by a conserved machinery consisting of compartment-specific v-SNAREs³ on transport vesicles and t-SNAREs on the target membrane (1–4). SNAREs are characterized by SNARE motifs, stretches of 60–70 amino acids, which contain heptad repeats with a central “0” layer and assemble into specific four-helix

bundles (5). The formation of SNAREpins, *trans* v-/t-SNARE complexes bridging two membranes, occurs in a zipper-like manner that starts at the membrane distal (N-terminal) end of the SNAREpins and proceeds toward the (C-terminal) membrane-spanning anchors of the SNAREs (6, 7). Zippering brings the two lipid bilayers in close apposition, finally resulting in membrane merger (2, 8). Thus, the energy required for membrane fusion is provided by the exergonic folding of the largely unstructured v- and t-SNARE proteins into stable four-helix bundles (2, 5, 9). Although SNAREs can be considered to be the minimal membrane fusion machinery, in the physiological cellular environment, an array of accessory proteins and lipids controls the spatial and temporal activity of SNARE proteins (10).

One class of accessory proteins, the SM (Sec1p/Munc18) proteins, directly bind to SNAREs, control their activity, and are required for membrane fusion *in vivo* (11–22). SM proteins contain about 600 amino acids, which are folded into an arch-shaped structure. At least two SNARE-binding modes have been described. In the first mode, the SM protein binds the t-SNARE component syntaxin in a “closed” conformation, in which the N-terminal three-helical Habc domain of syntaxin folds back on a part of the SNARE motif (23–25). In this conformation, syntaxin cannot bind its cognate SNARE partners (26). Binding of Munc18 to this closed conformation is also important for the transport of syntaxins from the endoplasmic reticulum to the plasma membrane and syntaxin stability (27–33). In the second mode, the SM protein binds t-SNAREs, SNAREpins, and fully assembled *cis* v-/t-SNARE complexes and contacts residues on the exposed surface of both the v-SNARE and t-SNARE (29, 34–40). In this binding mode, the SM protein likely assists SNAREpin organization and assembly (34, 41–43). Therefore, SM proteins can function as catalysts of SNARE complex formation, and hence the combination of the SM and SNARE proteins has been designated to be the universal fusion machinery (44). This terminology expands the concept of the SNAREs, representing the minimal components of the fusion machinery. Consistent with this notion, reconstituted fusion assays have revealed that defined SM proteins increase selectively the fusion of distinct v- and t-SNARE partners (41, 43).

Molecular binding sites contributing to the SM/SNARE interactions have been mapped to the N-terminal peptides of syntaxins, the Habc domains of syntaxins, the linker connecting the Habc domain with the SNARE motif, the syntaxin SNARE motifs, and cognate v-SNAREs (35, 36, 45–49). One of the best

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³ The abbreviations used are: SNARE, SNAP receptors; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SM, Sec1p/Munc18; β ME, β -mercaptoethanol; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl).

studied model systems is neurotransmitter release, which employs the v-SNARE VAMP2/synaptobrevin 2 (on synaptic vesicles), the t-SNAREs syntaxin 1, SNAP-25 (on the plasma membrane), and the cognate SM protein Munc18-1 (19, 50–52). Specific point mutations (L165A/E166A) in the linker of syntaxin 1 reduce the affinity for Munc18-1 by interfering with binding mode 1 and increase t-SNARE assembly (26). This result strongly suggests that this syntaxin 1 mutant mimics an “open” conformation. The transition from the syntaxin/SM conformation to the SNAREpin-SM complex apparently requires such an open syntaxin 1. Binding studies revealed the existence of an open syntaxin 1/Munc18-1 intermediate (29, 53). However, components and mechanisms favoring the closed to open transition still need to be characterized in detail. In living cells, this transition is facilitated by regulatory components, such as Munc13 and lipids (54–58). Interestingly, in *Caenorhabditis elegans*, the open syntaxin (L165A/E166A) can rescue the secretion defect observed in *unc-13* mutants, but cannot rescue an *unc-18* null mutant (18, 59). Together with recent studies, these results provide further *in vivo* support for the late acting (mode 2) function of Munc18 in SNAREpin formation/assembly (60).

The syntaxin 1 N-peptide appears to be essential for the Munc18-1 stimulation of membrane fusion both *in vitro* and *in vivo*, but its exact role is still debated, and controversial observations have been published (19, 29, 31, 36, 41, 53, 61). In a reconstituted liposome fusion assay containing preassembled t-SNARE complexes, the presence of the N-peptide favors membrane fusion (41, 62). In contrast, using the same components, but now in solution, the presence of the N-peptide inhibits the formation of a stable v-/t-SNARE complex in the presence of Munc18-1 (35). When UNC-18 mutants (F113R and L116K) that selectively abolish the N-peptide interaction were expressed in *unc-18* null *C. elegans*, the defect in regulated exocytosis could not be rescued, supporting the functional importance of the Munc18-1/syntaxin 1 N-peptide interaction (31, 53). Furthermore, the N-peptide inhibits neurotransmitter release in the calyx of Held synapses (37). However, in secretion-deficient PC12 cells, which lack both Munc18-1 and Munc18-2, Munc18-1 mutants (F115E and E132A) that impair the binding to the syntaxin N-peptide Munc18-1 rescue exocytosis to a large degree (32, 63). This result suggests a more subtle role of the syntaxin 1 N-peptide in dense core vesicle exocytosis.

To obtain further insights into the different syntaxin 1/Munc18-1 interaction modes and the role of the N-terminal syntaxin 1 peptide in this reaction cascade, we established a liposome fusion assay, which measures the assembly of the t-SNAREs on liposomes and the subsequent SNAREpin formation between the v- and t-SNARE liposomes. In such an assay, syntaxin 1 can adopt its stage-specific conformations, and the role of Munc18-1 can be studied at distinct steps of the fusion reaction using lipid mixing as the ultimate readout signal.

EXPERIMENTAL PROCEDURES

DNA Constructs—DNA constructs were made using standard genetic manipulations. Phusion polymerase for PCR, restriction enzymes, and DNA ligases were obtained from New

England Biolabs. The following bacterial expression vectors were used: pGEX-4T1 (Stratagene) and pET24 and pET28 (Novagene). Mutations were introduced using the QuikChange II site-directed mutagenesis kit (Stratagene). Bacterial expression plasmids encoding N-terminal His₆-tagged SNAP-25 (pFP247), C-terminal His₆-tagged VAMP2 (pTW2), rat syntaxin 1A (pTW20), and GST-tagged VAMP8 (pKL12) have been described previously (2, 64, 65). Constructs encoding the C-terminal His₆-tagged Munc18-1 (pYS6), the open conformation (L165A/E166A) of syntaxin 1A (pYS1), and the following C-terminal His₆-tagged syntaxin 1A variants: wild type (wt) (pYS2), open conformation (pYS4), N-terminal deletion (d24) (pSK23), and the point mutation (L8A) (pSK21) are described in the [supplemental material](#).

Protein Expression and Purification—Recombinant proteins were expressed in the *Escherichia coli* strain BL21(DE3) (Stratagene). The culture media were supplemented with the appropriate antibiotics (50 µg/ml kanamycin or 100 µg/ml ampicillin). Cells were grown at 37 °C in 12 liters of LB media to an *A*₆₀₀ of 0.8. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside. t-SNARE complexes were formed by cotransforming pFP247 (His₆-SNAP-25) together with either pTW20 (syntaxin 1 (WT)) or pYS1 (syntaxin 1 (open conformation)). t-SNARE complex and v-SNARE expressions were induced at 37 °C for 3 h. Syntaxin 1 constructs and Munc18-1 were induced at 16 °C overnight. Cells were collected by centrifugation and washed once with PBS, resuspended in breaking buffer, snap-frozen in liquid nitrogen, and stored at –80 °C. The breaking buffer for the t-SNARE complex was composed of 25 mM HEPES/KOH, pH 7.4, 400 mM KCl, 10% glycerol, 2% Triton X-100, 30 mM imidazole and freshly added 2 mM β-mercaptoethanol (βME). For syntaxin 1 and Munc18-1 purification, the salt concentrations were reduced to 150 mM KCl. The bacterial pellets were rapidly thawed in a final buffer volume of 300 ml, containing 2 mM βME and a protease inhibitor mixture (final concentrations: leupeptin (1.5 µg/ml), antipain (2.5 µg/ml), turkey trypsin inhibitor (25 µg/ml), benzamidin (12.5 µg/ml), Pefabloc SC (6.25 µg/ml), aprotinin (1.25 µg/ml), chymostatin (5 µg/ml), and pepstatin (2.5 µg/ml)). Cells were lysed by one pass at 18,000 p.s.i. through a Microfluidizer M110L (Microfluidics). Insoluble material was removed by ultracentrifugation for 60 min at 40,000 rpm at 4 °C in a 45Ti rotor (Beckman Coulter).

50 ml of the supernatants containing His₆-tagged proteins were incubated for 1 h at 4 °C with 1.5 ml of nickel-nitrilotriacetic acid beads (Qiagen). The beads were washed two times with breaking buffer and two times with buffer A (25 mM HEPES/KOH, pH 7.4, 100 mM KCl, 10% (w/v) glycerol, 2 mM βME) containing 30 mM imidazole and 1% Triton X-100. Beads were packed into a chromatography column and extensively washed with buffer A containing 50 mM imidazole and 1% *n*-octyl-β-D-glucoside. Proteins were eluted from the nickel-nitrilotriacetic acid resin with a gradient from 50 to 500 mM imidazole in 25 mM HEPES/KOH, pH 7.4, 100 mM KCl, 10% glycerol, 1% (w/v) *n*-octyl-β-D-glucoside, and 2 mM βME. SNAP-25 and Munc18-1 were purified in the absence of detergent. SNARE proteins were purified as described previously (2, 65). Munc18-1 was directly eluted in buffer A containing 500 mM

imidazole and dialyzed against buffer A followed by an ultracentrifugation step at 50,000 rpm for 30 min at 4 °C in a TLA55 rotor (Beckman Coulter). The supernatant was again dialyzed against buffer A, and protein aggregates were removed by ultracentrifugation as mentioned above. GST-VAMP8 was purified on glutathione beads, eluted by thrombin cleavage, and further purified using Mono S-Sepharose chromatography (GE Healthcare) (65). VAMP2 and SNAP-25 were further purified using Mono Q and Mono S chromatography (GE Healthcare), respectively.

Protein concentrations were determined by SDS-PAGE and Coomassie Blue staining using defined amounts of BSA as the protein standard. Protein amounts were quantitated using the ImageJ software (National Institutes of Health).

Protein Reconstitution into Liposomes—Unless otherwise stated, all lipids were purchased from Avanti Polar Lipids. SNAREs were reconstituted into liposomes as described previously (2). However, the following modifications were made. The acceptor lipid mixture used to reconstitute syntaxin 1 and t-SNAREs was composed of 35 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 25 mol % 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 25 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, 5 mol % 1- α -phosphatidylinositol (liver and bovine), and 10 mol % cholesterol. The donor lipid mix, used to reconstitute VAMP2/VAMP8, was composed of 82 mol % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 15 mol % 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 1 mol % 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE), and 2 mol of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (rhodamine-PE). To quantitate lipid reconstitution efficiency, trace amounts of 1,2- ^3H -dipalmitoylphosphocholine (Amersham Biosciences) were added. Liposomes were formed by diluting the *n*-octyl- β -D-glucoside below the critical micelle concentration, followed by flow dialysis, and flotation of the reconstituted liposomes in a Nycodenz gradient as described previously (2). The protein to lipid ratios obtained after reconstitution are mentioned in the text.

Light Scattering—To determine the hydrodynamic size of the reconstituted liposomes a Zetasizer 1000HS (Malvern Instruments) was employed. Light scattering was measured at 633 nm, and the mean diameters of the vesicles were determined using the analysis software supplied by Malvern Instruments. Vesicle mean diameters are based on the peak analysis by intensity.

Fusion Assays—Assays were performed in white 96-microwell FluoroNunc plates (Nunc). Typically, 5 μl of fluorescently labeled v-SNARE vesicles (containing either VAMP2 or VAMP8, ~ 6 nmol of lipid) were mixed with 30 μl of t-SNARE- or syntaxin-liposomes (~ 157 nmol lipid), and fusion was measured in buffer A in the absence or presence of additional components in a final volume of 70–80 μl . Specific preincubation and order of addition procedures are described under “Results.” Briefly, preincubation steps occurred usually in 500- μl standard reaction tubes at the indicated temperatures. Then the probes were transferred to and mixed in a preheated 96-well plate, and the fluorescence measurements were immediately

started in the prewarmed fluorescent microplate reader (Fluoroskan Ascent FL, Thermo Scientific). NBD fluorescence was detected with filters at 460 nm (excitation) and 538 nm (emission) and monitored at 1-min intervals. After 2 h at 37 °C, 10 μl of a 2.5% (w/v) *n*-dodecyl- β -D-maltoside solution was added to terminate the reaction and to allow maximum fluorescence dequenching. Fluorescence measurements were normalized by setting the lowest NBD fluorescence signal to zero and by setting the NBD fluorescence after *n*-dodecyl- β -D-maltoside lysis to 100% as described previously (2). The maximum fusion rates within the first 30 min of the fusion reaction were used to determine the initial fusion rates and to calculate the inhibition/stimulation efficiencies relative to the fusion reaction containing wild type syntaxin 1 in the absence of Munc18-1. The statistical analyses includes at least three independent experiments.

RESULTS

Syntaxin 1 Liposomes Fuse with VAMP2 Liposomes in a SNAP-25-dependent Manner—To test the role of Munc18-1 in t-SNARE complex assembly in the membrane environment and to monitor subsequent membrane fusion, recombinant syntaxin 1 was expressed in bacteria, purified, and reconstituted into liposomes. To mimic the SNARE density in physiological membranes, VAMP2 was reconstituted at a protein to lipid ratio of about 1:250, corresponding to the VAMP2 density in synaptic vesicles (66). Syntaxin 1 was reconstituted into liposomes at a protein to lipid ratio of $\sim 1:3000$. However, because of some variations in the reconstitution efficiencies of the different syntaxin 1 constructs, the syntaxin 1 to lipid ratios covered a range of 1:2750–1:4500. When different syntaxin constructs were directly compared, liposomes containing similar protein to lipid ratios were employed, and in every case aliquots of the fusion reactions were analyzed by SDS-PAGE and Coomassie Blue staining. Dynamic light scattering revealed that VAMP2- and syntaxin 1-liposomes had mean diameters of about 80 and 130 nm, respectively. Liposome aggregates were not detectable. Taking account of these size estimates, ~ 17 – 28 syntaxin 1 molecules would be exposed on the surface of a 130-nm liposome, whereas VAMP2 liposomes would contain about 120 surface-exposed v-SNAREs. To obtain robust fusion signals, v-SNARE liposomes were incubated with a 10-fold molar excess of syntaxin 1 liposomes. Thus, at low temperature, which usually blocks/slows down lipid mixing, an average of 10 syntaxin 1 liposomes would bind to a single v-SNARE liposome, containing 120 VAMP2 molecules. Making this assumption, 12 SNAREpins would theoretically be available per fusion site. Because 1–8 SNAREpins appear to be sufficient to drive membrane fusion, the average number of available SNAREpins per docking/fusion site will not become a rate-limiting factor in the fusion assay (67–69). Membrane fusion was measured by a well established lipid-mixing assay based on fluorescence dequenching (2, 70). When donor VAMP2 liposomes, which contain a quenched pair of fluorescently labeled lipids (rhodamine-PE, NBD-PE), fuse with unlabeled acceptor syntaxin 1 liposomes, the fluorophores are diluted, and the NBD fluorescence increases. Fig. 1A shows that syntaxin 1 liposomes do not fuse to a significant degree with VAMP2 liposomes in the

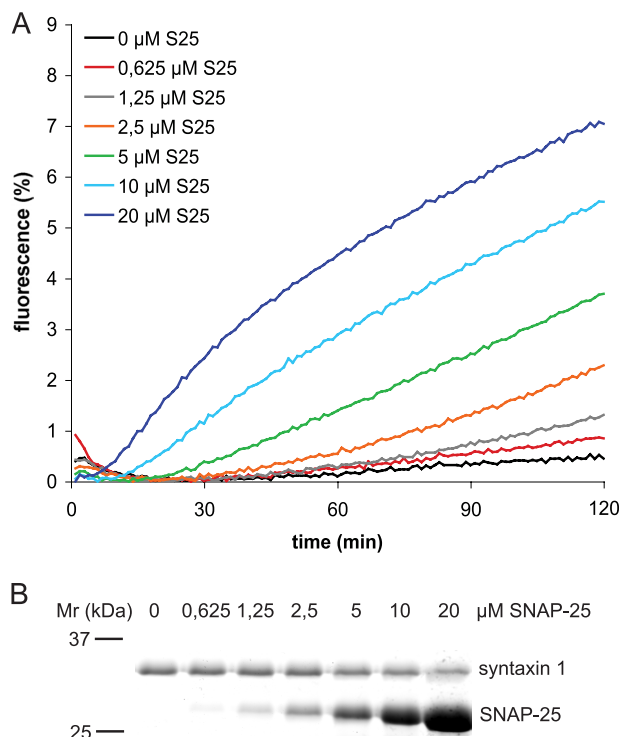


FIGURE 1. Fusion of VAMP2 and syntaxin 1 liposomes requires SNAP-25. A, 30 μ l of syntaxin 1 liposomes were preincubated with increasing amounts of soluble SNAP-25 (S25) and 5 μ l of v-SNARE liposomes containing rhodamine- and NBD-labeled lipids for 1 h on ice, in a total volume of 70 μ l (see also "Experimental Procedures"). Samples were transferred into a prewarmed microwell plate, and lipid mixing was monitored at 37 $^{\circ}$ C. After 2 h, *n*-dodecyl- β -D-maltoside was added, and the fusion signals were normalized to the maximum NBD fluorescence as described under "Experimental Procedure." B, 20% of the fusion reactions shown in A were separated by SDS-PAGE, and the proteins were stained by Coomassie Blue.

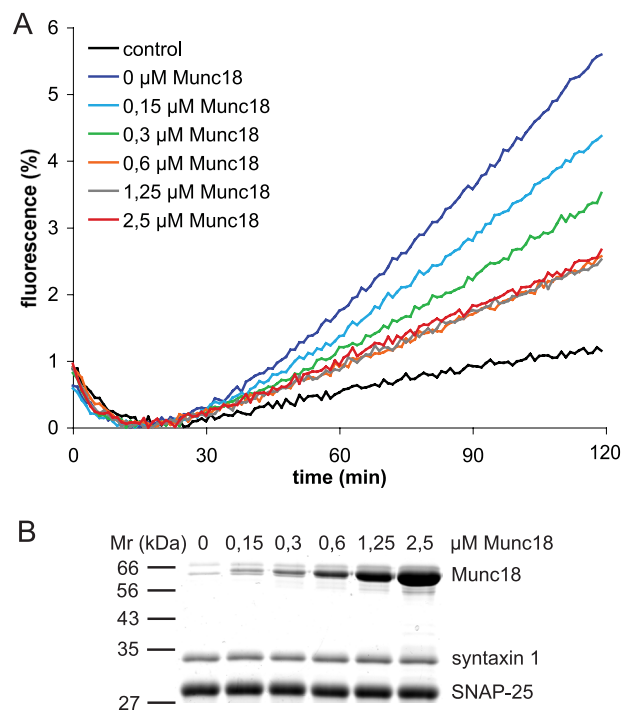


FIGURE 2. Munc18-1 inhibits syntaxin 1 liposome fusion. A, 30 μ l of syntaxin 1 liposomes were preincubated with the indicated amounts of Munc18-1 in a final volume of 72 μ l at room temperature for 30 min. Reaction vials were transferred into an ice bath and incubated with SNAP-25 (4-fold molar excess over syntaxin 1) for 1 h, and subsequently 5 μ l of v-SNARE liposomes were added (final volume 80 μ l). The control incubation lacks SNAP-25. Samples were transferred into a prewarmed microwell plate, and fusion was measured at 37 $^{\circ}$ C. Samples were analyzed as described in the legend to Fig. 1 and "Experimental Procedures." B, 20% of the fusion reactions shown in A were separated by SDS-PAGE, and the proteins were stained by Coomassie Blue.

absence of SNAP-25. Thus, membrane fusion depends on the presence of SNAP-25, and increasing concentrations of soluble SNAP-25 raise both the initial rate and the final extent of lipid mixing (Fig. 1A). Efficient fusion requires a significant molar excess of SNAP-25 over membrane-embedded syntaxin 1 (Fig. 1B). We noticed that t-SNARE complex formation in the membrane environment is less efficient than the t-SNARE complex formation in solution suggesting that membrane-embedded syntaxin 1 is less reactive. Therefore, the fusion efficiencies are lower than those obtained with liposomes containing already preassembled t-SNARE complexes at similar protein to lipid ratios (data not shown). These results suggest that the formation of productive t-SNARE complexes is the rate-limiting step in this liposome fusion assay.

Munc18-1 Inhibition of Liposome Fusion Requires the Closed Conformation of Syntaxin 1 but Occurs Independently of the Syntaxin 1 N-peptide Interaction—Because t-SNARE complex formation is the rate-limiting step, it would be expected that Munc18-1, which stabilizes the closed conformation of syntaxin 1, should block fusion. Indeed, a 30-min preincubation of syntaxin 1 liposomes with increasing amounts of Munc18-1, followed by the addition of SNAP-25, significantly inhibits membrane fusion (Fig. 2A). Even preincubation of the syntaxin 1 liposomes with Munc18-1 for a few minutes was sufficient to obtain the inhibition (data not shown), because the formation of syntaxin 1-Munc18-1 complexes is fast and efficient com-

pared with t-SNARE complex assembly. Control experiments in the absence of SNAP-25 revealed that Munc18-1 does not affect the fluorescent signal (data not shown). Maximum inhibition was reached when Munc18-1 and syntaxin 1 were present at equimolar amounts (compare lanes in Fig. 2, A and B). Inhibition was efficient (80%) (see also Fig. 3D) but not complete, because the syntaxin 1 liposomes may contain a syntaxin pool that is binding-competent for SNAP-25 but not for Munc18-1. This syntaxin pool could contain syntaxin 1 in the open conformation.

To test if a functional syntaxin 1 N-peptide is required for the Munc18-1-mediated inhibition, a syntaxin 1 construct lacking the N-terminal 24 amino acids (d24) and a syntaxin 1 point mutation (L8A) impairing the interaction of Munc18-1 with the syntaxin 1 N-peptide were reconstituted into liposomes. These mutants displayed slightly reduced membrane fusion kinetics, but the inactivation of the N-peptide did not abolish the inhibitory function of Munc18-1 in the liposome fusion assay (Fig. 3A and supplemental Fig. S1). Fig. 3D shows a comparison of the initial kinetics of the fusion reactions relative to the wild type syntaxin 1.

Next we analyzed how the open conformation of syntaxin 1 (L165A/E166A), which is characterized by a reduced Munc18-1 affinity and an impaired binding mode 1, affects membrane fusion in the presence of Munc18-1 (26). In the presence of the open conformation of syntaxin 1, Munc18-1 did

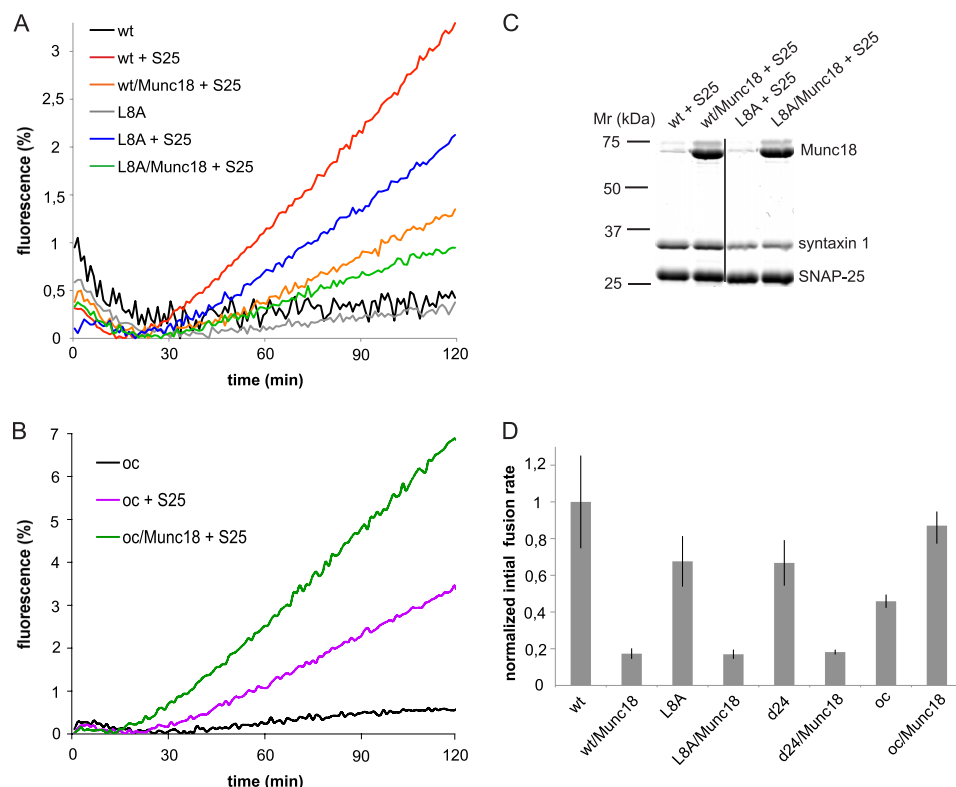


FIGURE 3. Munc18-1 inhibition requires a closed conformation of syntaxin 1 but occurs independently of the syntaxin 1 N-peptide. 30 μ l of liposomes containing either wild type syntaxin (wt) or mutant syntaxin (L8A) (A) or syntaxin (B) (open conformation (oc)) were preincubated in the absence or presence of Munc18-1 (2-fold molar excess over syntaxin 1) in a final volume of 60 μ l at room temperature for 30 min. Reactions were transferred into an ice-bath and incubated with 5 μ l of SNAP-25 (4-fold molar excess over syntaxin 1) for 1 h. Control samples (wt and L8A) lacked SNAP-25 but contained buffer. Reactions were transferred into a preheated microwell plate, and fusion was started by adding 5 μ l of v-SNARE liposomes to all samples (total volume of 70 μ l). C, 20% of the fusion reactions shown in A were separated by SDS-PAGE, and the proteins were stained by Coomassie Blue. Lanes from the same gel were cropped as indicated. D, bar graph showing the effect of Munc18-1 on the initial rates of fusion reactions containing various syntaxin 1 constructs. For comparison, the initial fusion rate of the reaction containing wild type syntaxin 1 and VAMP2 liposomes was set to 1 and used to normalize the other reactions. Error bars represent the mean \pm S.E. The various syntaxin liposomes were preincubated with Munc18-1; subsequently SNAP-25 was added, and finally fusion was started by the addition of VAMP2 liposomes as described in A and B.

not show any inhibitory effects; it even stimulated liposome fusion by a factor of ~ 2 (Fig. 3, B and D). The stimulation suggests that an interaction of Munc18-1 with the open syntaxin increases SNAREpin formation/assembly. Unexpectedly, Fig. 3D also demonstrates that the open conformation of syntaxin shows a 2-fold lower fusion activity than wild type syntaxin 1. The open syntaxin might form oligomers or increased amounts of t-SNARE complexes, which contain syntaxin1/SNAP25 at a molecular ratio of 2:1, which are known to inefficiently form SNAREpins (7). Taken together, the data clearly demonstrate that the inhibitory function of Munc18-1 requires syntaxin 1 in its closed conformation but occurs independently of the Munc18-1/syntaxin 1 N-peptide interaction.

Preincubation of Munc18-inhibited Syntaxin 1 Liposomes in the Presence of SNAP-25 and VAMP2 Liposomes Stimulates Lipid Mixing—Previous experiments have shown that Munc18-1 stimulates liposome fusion when the liposomes contain preassembled t-SNAREs (41). This stimulation strictly required a preincubation of Munc18-1 with both the v-SNARE and t-SNARE liposomes under nonfusogenic conditions and was VAMP2-specific. Thus, the issue raises the following question. Is the inhibited state of the syntaxin 1·Munc18-1 complex released by a subsequent incubation with both SNAP-25 and

VAMP2 liposomes? To test this point, we changed the previous incubation regime; syntaxin 1 liposomes were preincubated with Munc18-1 for 30 min at room temperature as before, but now SNAP-25 and VAMP2 liposomes were added simultaneously, incubated for 1 h on ice, which inhibits lipid mixing, and subsequently warmed up to 37 $^{\circ}$ C to start membrane fusion (compare incubation schemes in Fig. 4A). Remarkably, the preincubation in the presence of VAMP2 liposomes was sufficient to reverse the inhibitory effect of Munc18-1, and more importantly Munc18-1 stimulated fusion (Fig. 4B). The analysis of the initial kinetics shows that Munc18 stimulates liposome fusion by a factor of 5.5, which is comparable with the Munc18 stimulation observed with preassembled t-SNAREs (Fig. 4D) (41). Thus, the closed Munc18-1·syntaxin 1 complex, which based on our previous experiments, was in an inhibited state (Fig. 2), was able to switch to an open conformation, allowing SNAREpin formation. Munc18-1, which now interacts with VAMP2, apparently stabilizes the newly formed SNAREpins and/or provides additional force to favor SNAREpin zippering and membrane fusion. To test if this reaction indeed requires the dual interaction of Munc18-1 with the v- and t-SNARE, fusion reactions were also performed with VAMP8 liposomes. When VAMP8 liposomes were used, neither the Munc18-1 inhibition was released nor was the stimulation observed (Fig.

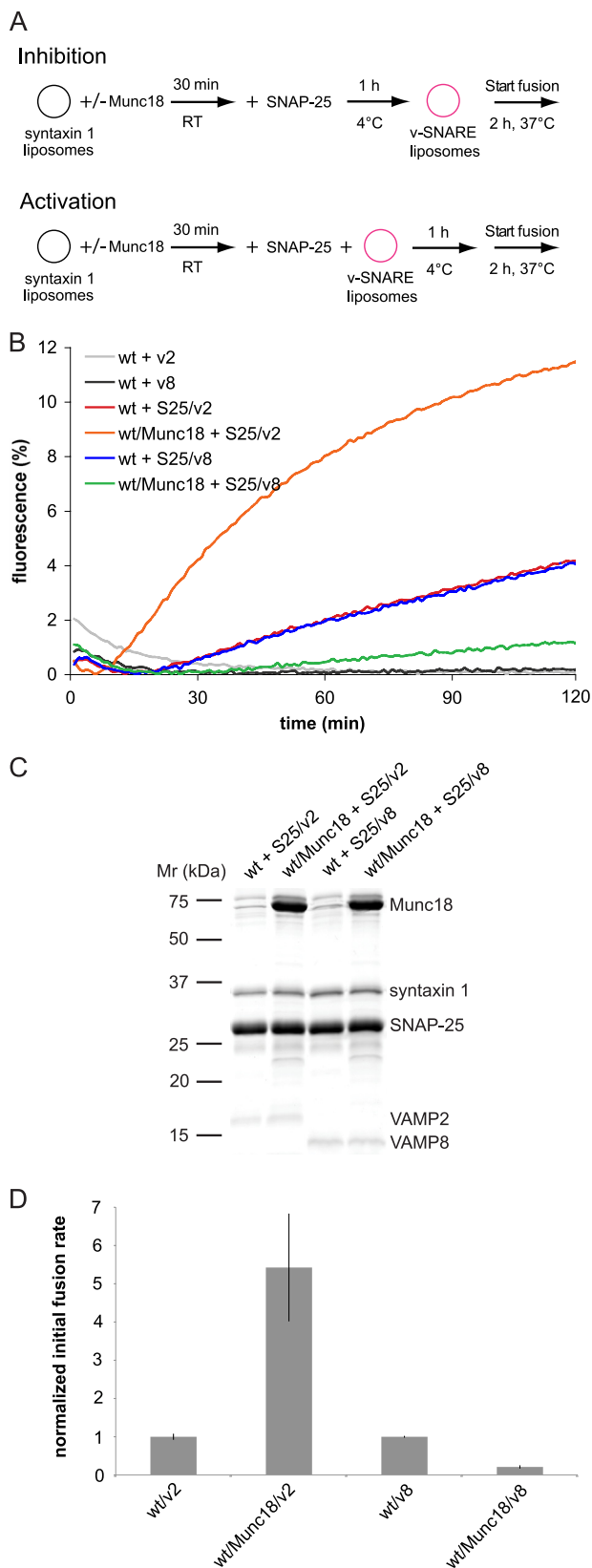


FIGURE 4. Preincubation of Munc18-1-inhibited syntaxin 1 liposomes with VAMP2 liposomes in the presence of SNAP-25 at low temperature stimulates membrane fusion in a v-SNARE-dependent manner. *A*, scheme illustrates the distinct incubation steps and the order of addition of the reaction partners. Note, compared with previous experiments (*upper panel*), the v-SNARE liposomes were now added together with SNAP-25 to the Munc18-1 inhibited syntaxin 1 liposomes and incubated for 1 h at 4°C

(*lower panel*). *B*, 30 μ l of syntaxin 1 (wt) liposomes were incubated according to the scheme shown in the *lower panel* of *A* with the indicated components and either with 5 μ l of VAMP2 liposomes (v2) (lipid to protein ratio 350:1) or 5 μ l of VAMP8 liposomes (v8) (lipid to protein ratio 275:1) in a total volume of 70 μ l. Control samples lacked SNAP-25. Fusion was monitored and analyzed as described before. *C*, 20% of the fusion reactions shown in *B* were separated by SDS-PAGE and the proteins were stained by Coomassie Blue. *D*, comparison of the initial fusion rates of reactions containing wild type syntaxin 1 and either VAMP2 or VAMP8 liposomes in the absence or the presence of Munc18-1. The initial fusion rate of the reaction containing wild type syntaxin 1 liposomes and VAMP2 liposomes was set to 1 and used to normalize the other reactions. Error bars represent the means \pm S.E.

Thus, the inhibition release and stimulation need a productive Munc18-1/VAMP2 interaction.

Syntaxin 1 N-peptide/Munc18-1 Interaction Is Required to Stimulate Liposome Fusion—To test if the stimulatory function requires an interaction of Munc18-1 with the syntaxin 1 N-peptide, the syntaxin 1 constructs containing the L8A and the d24 mutations were used in the fusion assay. Both mutations significantly reduced the Munc18-1-dependent stimulation, indicating that the stimulatory effect requires an interaction of Munc18-1 with the syntaxin 1 N-peptide (Fig. 5, *A* and *D*, and supplemental Fig. S2). Interestingly, the syntaxin 1 N-peptide seems not to be necessary to release the Munc18-1 inhibition. We also tested the open conformation of syntaxin 1, which did not show any Munc18-1-dependent inhibition in the t-SNARE complex assembly assay. As expected, the open syntaxin 1 was characterized by a dramatic stimulation, clearly visible in the initial fusion rates (supplemental Fig. S3). Interestingly, when VAMP8 liposomes were used, we observed a small but reproducible stimulation by Munc18-1 (supplemental Fig. S3). Hence, independent of a VAMP2 interaction, Munc18-1 might further support membrane fusion by increasing the number of t-SNARE complexes containing open syntaxin 1. To support the conclusion that t-SNARE complex formation contributes a rate-limiting step in the overall fusion reaction, we bypassed the t-SNARE complex assembly process by reconstituting preassembled t-SNAREs containing either the wt or open syntaxin 1 into liposomes and analyzed them in the fusion assay. In these experiments, the fusion kinetics of the wild type and open syntaxin 1 in the presence of Munc18-1 did not differ significantly (supplemental Fig. S4). Thus, when t-SNARE complexes have formed, an open syntaxin 1 conformation does not contribute additional functions to the subsequent reactions in the reconstituted system.

DISCUSSION

Here, we have resolved the different functional properties of Munc18-1 and syntaxin 1 at distinct stages of SNARE complex assembly in a reconstituted fusion assay, which analyzes SNARE complex formation in a membrane environment and measures lipid mixing as the ultimate functional readout. Our experiments reveal that the inhibitory effect of Munc18-1 depends on a closed conformation of syntaxin 1 but does not require the N-peptide of syntaxin 1. The observation that Munc18-1 inhibits t-SNARE complex assembly in a lipid environment is consistent with previous experiments demonstrating the inhibitory mode in solution (71). Because we used soluble SNAP-25, which lacks the post-translational palmitoyl

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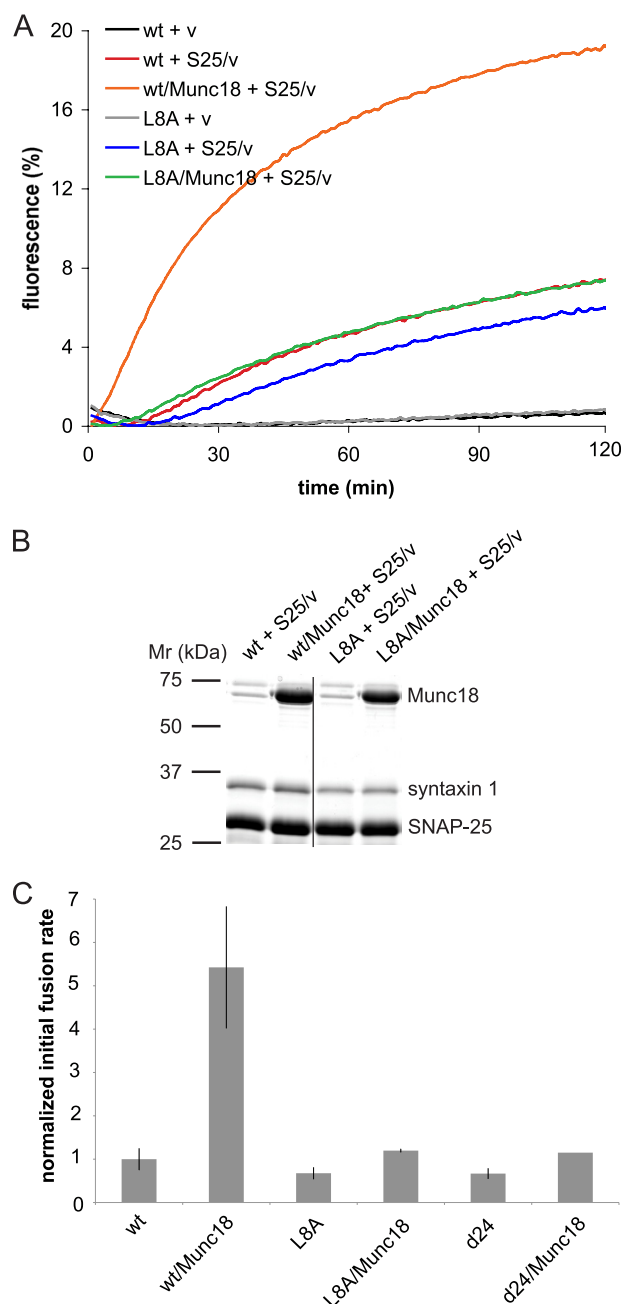


FIGURE 5. Syntaxin 1 N-peptide contributes to Munc18-1 mediated stimulation of membrane fusion. *A*, 30 μ l of syntaxin (wt) liposomes or syntaxin (L8A) liposomes were incubated in the absence or the presence of Munc18-1 (2-fold molar excess over syntaxin 1) at room temperature for 30 min in a total volume of 50 μ l. Reactions were transferred onto ice and a 3-fold molar excess of SNAP-25 and 5 μ l of v-SNARE liposomes were added and incubated for an additional hour in a final volume of 70 μ l. Samples were transferred into a preheated microwell plate, and fusion was monitored as described before. *B*, 20% of the fusion reactions shown in *A* were separated by SDS-PAGE, and the proteins were stained by Coomassie Blue. Lanes from the same gel were cropped as indicated. *C*, comparison of the initial rates of fusion reactions containing either wt, or L8A, or d24 syntaxin 1 liposomes in the absence or presence of Munc18-1. The initial fusion rate of the reaction containing wild type syntaxin 1 liposomes and VAMP2 liposomes was set to 1 and used to normalize the other reactions. All reactions contained SNAP-25. Error bars represent the means \pm S.E.

modifications, we cannot exclude that t-SNARE complexes might form more efficiently on liposomes in the presence of palmitoylated SNAP-25. However, the presence of palmitoyl membrane anchors in SNAP-25 should not affect the inhibitory

Munc18-1/syntaxin 1 interaction that is characterized by distinct binding revealed in the crystal structure of the Munc18-1-syntaxin 1 complex (24). Remarkably, the simultaneous preincubation of the Munc18-1-inhibited syntaxin 1 liposomes with SNAP-25 and VAMP2 liposomes at low temperature (nonfusogenic conditions) releases the Munc18-1 inhibition, and Munc18-1 is converted into a stimulator. The inhibition release and the stimulation depend on the specific Munc18-1/VAMP2 interaction. Thus, at steady state an inhibitory Munc18-1-syntaxin 1 complex dominates in the presence of SNAP-25, and only a small fraction of t-SNARE complexes might form. In the presence of v-SNARE liposomes, this small fraction of t-SNARE complexes can bind the v-SNARE resulting in SNAREpins that still need to zipper up in the membrane proximal region. Because VAMP8 liposomes are not sufficient to efficiently relieve the inhibitory function of Munc18-1, it becomes apparent that the specific interaction of Munc18-1 with VAMP2 is required to further drive the reaction. In the simplest model, Munc18-1 binds VAMP2 in partially assembled SNAREpins, thereby enhancing SNAREpin stability/assembly and membrane fusion (41). Thus, the small fraction of SNAREpins, which forms at steady state, would be constantly consumed by the action of Munc18-1. Alternatively, VAMP2 might function at an earlier stage before it binds its cognate t-SNARE partners. In an initial step, VAMP2 could interact with the Munc18-1-syntaxin 1 complex, relieve the inhibitory conformation, and subsequently SNAP-25 binding would follow. The exact order in which VAMP2 interacts with Munc18-1 and its cognate t-SNAREs still remains open.

A recent study showed that VAMP2 itself and VAMP2 as part of the SNARE four-helix bundle can compete with the syntaxin 1 Habc domain for binding to the central cavity of Munc18-1, thereby suggesting a potential molecular reaction mechanism (72). A model emerges in which VAMP2 contributes to the displacement of the Habc domain from the central cavity of Munc18-1 or blocks the reassociation of Munc18-1 with the Habc domain. Mapping of the binding site revealed that Munc18-1 interacts with the membrane proximal region of VAMP2 (72). Based on FRET experiments VAMP2 likely binds domain 3a of Munc18-1 (72). In addition, structural analysis showed that domain 3a can adopt a helical structure, which is compatible with a direct interaction with the helical SNARE motif of syntaxin 1 (73). Thus, upon relief of the Habc domain inhibition, the central cavity of Munc18-1 and likely domain 3a will be available to bind the SNARE motifs within the SNAREpin. In this binding mode, Munc18-1 stimulates SNAREpin assembly and membrane fusion. Indeed, *in vitro* fusion experiments using preassembled t-SNAREs showed that the central cavity of Munc18-1 contains critical amino acids that are required to stimulate fusion (62). Point mutations within the Habc domain that impair the binding to the central cavity of Munc18-1 or removal of the entire Habc domain, excluding the N-peptide, do not abolish Munc18-1-dependent fusion stimulation *in vitro* (62). In contrast, the syntaxin 1 N-peptide/Munc18-1 interaction is required to stimulate membrane fusion (41, 62). Although the exact function of the syntaxin 1 N-peptide still remains unclear, recent structural analyses suggest that it can alter the conformation of Munc18-1 (73). Our

functional studies show that the release of the Munc18-1 inhibition indeed requires VAMP2 but can occur independently of the syntaxin 1 N-peptide. Interestingly, the Munc18-1/syntaxin 1 N-peptide interaction is also controlled by phosphorylation, which inhibits regulated exocytosis (61). In general, dependent on the intracellular transport step, the physiological requirements for syntaxin N-peptides and the affinities of distinct Munc18 homologs for syntaxin N-peptides, Habc domains, and SNARE complexes can vary significantly (74).

A previous study showed that the deletion of the syntaxin 1 N-peptide (first 24 amino acids) permits v-/t-SNARE complex formation in the presence of Munc18-1, suggesting an inhibitory role for the syntaxin 1 N-peptide/Munc18-1 interaction (35). This inhibitory role of the N-peptide in SNARE complex assembly seems to contradict the stimulatory role of the N-peptide in membrane fusion. However, the different experimental approaches provide an explanation. In one set of experiments, the assembly of cytoplasmic SNARE domains was measured (35). These experiments also show that the affinity of Munc18-1 for the cytoplasmic domain of syntaxin 1 (K_d 1.4 ± 0.3 nM) drops in the absence of the N-peptide (K_d 8.1 ± 1.0 nM). The binding of Munc18-1 to the N-terminal regulatory domain (amino acids 1–179) is significantly weaker (K_d 693.9 ± 84.2 nM). Thus, the absence of the N-peptide further reduces the affinity of Munc18-1 for the Habc domain ((35) and data not shown). Because the Habc domain apparently functions as an inhibitor, a weaker Munc18-1 interaction (in the absence of the syntaxin 1 N-peptide) would reduce its inhibitory function, thus allowing *cis* SNARE complex formation as observed by Burkhardt *et al.* (35). The other sets of experiments analyze full-length SNAREs in their cellular environment or reconstituted into liposomes, measure SNAREpin assembly, and use membrane fusion as a functional readout system. In such assays, other functions for the N-peptide become apparent. Already, the membrane environment adds different constraints. For example, in solution, Munc18-1 shows only a weak interaction with assembled v-/t-SNARE complexes that lack the syntaxin N-peptide (41). Upon reconstitution of assembled v-/t-SNARE complexes into liposomes, the presence or absence of the N-peptide hardly affected Munc18-1 binding to the v-/t-SNARE liposomes (41). In addition, our experiments indicate that t-SNARE complex formation in liposomes differs considerably from t-SNARE complex formation in solution, consistent with a role of lipids in SNARE complex assembly (75, 76). It is also worth mentioning that the binding of Munc18-1 to membrane-embedded syntaxin 1 could significantly change the functional state of syntaxin 1 in such a manner that an inherently inactive pool of syntaxin might be shifted into a more reactive state (77). Among other possibilities, Munc18-1 could activate syntaxin 1 oligomers or clusters (78, 79). Our observation that Munc18-1 weakly stimulates the fusion of liposomes containing open syntaxin 1, independent of the specific v-SNARE, suggests that Munc18-1 is able to increase the pool of reactive syntaxin 1 molecules. We also noted that upon further reducing the syntaxin 1 to lipid ratio, which coincides with an overall reduction of the fusion signal, Munc18-1 stimulation became less dependent on the syntaxin 1 N-peptide. At such low syntaxin 1 copy numbers, the reactive syntaxin 1 molecules

per liposomes could become limiting, changing the rate-limiting step and additional functions of Munc18-1 might now dominate the overall reaction. Low protein to lipid ratios in the syntaxin liposomes make the assay in particular sensitive for changes in the functional state of the syntaxin 1 population. In reactions using already preassembled t-SNAREs (bypassing t-SNARE complex assembly), such an additional Munc18-1 activity has not been observed (62).

In conclusion, by probing the function of Munc18-1 in a reconstituted fusion assay, it is possible to assign distinct Munc18-1/SNARE interactions to different steps of the reaction cascade that mediates membrane fusion *in vivo*. Remarkably, under the experimental conditions employed, an external factor that releases the Munc18-1 inhibition is not strictly required, indicating that Munc18-1, syntaxin 1, SNAP-25, and VAMP2 are the core components of this off/on switch. Munc18-1 inhibits syntaxin 1 to prevent unspecific SNARE complex assembly and membrane fusion. The binding of Munc18-1 to VAMP2 then provides a basic switch to convert the inhibition into a compartment-specific stimulation. *In vivo* experiments demonstrate that additional factors such as lipids and Munc13 play an important role in syntaxin activation, suggesting that at least in the case of regulated exocytosis additional regulatory components have been added to further control the universal fusion machinery. We expect that variations of the reconstitution assay in combination with careful kinetic studies will be suitable to identify and characterize such factors controlling the Munc18-1 switch.

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